J. Membrane Biol. 48, 141–166 (1979)

Isolation and Separation of Toad Bladder Epithelial Cells

M. Rossier, B.C. Rossier, J. Pfeiffer, and J.P. Kraehenbuhl*

Institute of Pharmacology and Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland

Received 13 November 1978; revised 1 March 1979

Summary. The epithelium of the urinary bladder of *Bufo marinus* is composed of 5 cell types, i.e., granular (Gr), mitochondria-rich (MR) and goblet (G) cells which face the urinary lumen, microfilament-rich (MFR) and undifferentiated cells (Un) located basally. The epithelium was dissociated by collagenase and EGTA treatment. Fractionation of dispersed cells by isopycnic centrifugation on dense serum albumin solutions yielded 4 fractions: (i) a very light fraction ($\bar{\rho} \simeq 1.025$) enriched in MR and MFR cells; (ii) a light fraction ($\bar{\rho} \simeq 1.045$) enriched in vacuolated Gr cells; (iii) a heavy fraction ($\bar{\rho} \simeq 1.065$) composed essentially of aggregated Gr cells, and (iv) a pellet ($\bar{\rho} \simeq 1.085$) enriched in G and undifferentiated cells. Recoveries were based on cell counts and DNA measurements. DNA content per cell was 13.2 pg $\pm$ 0.9 ($n=37$). From 1 g fresh tissue, $62 \pm 5 \times 10^6$ ($n=10$) cells were recovered before isopycnic centrifugation of which about 70% excluded Trypan blue. After centrifugation, 90 to 95% of the cells excluded the vital dye and $\sim 3^9 \times 10^6$ cells were recovered from the gradient. Cell metabolism in each fraction was estimated by oxygen consumption measurements in absence or presence of ouabain, acetazolamide, and dinitrophenol. The consumption was threefold higher in the very light and light fractions when compared to the heavy and pellet fractions. Ouabain sensitive oxygen consumption (QO_2) represented 12 to 35% of the total O_2 consumption depending on the cell fraction, and acetazolamide sensitive QO_2 varied from -0.8% in the heavy fractions to 20% in the lighter fractions. DNP increased QO_2 in all fractions by 20 to 50%. Finally, the cells were able to reaggregate and form junctional complexes upon addition of calcium to the medium.

The urinary bladder of the toad *Bufo marinus* has been used as an experimental model for studying the transepithelial transport of Na^+ [15], H^+ [20], water and solutes [16]. In many respects, it represents the phylogenetic and functional analog of collecting tubules in the mammalian kidney [22]. Moreover, this model has contributed to our understanding of the subcellular mechanism of action of hormones such as aldosterone [6] or vasopressin [9]. The bladder has also been useful in

* *Address and to whom reprint requests should be made:* Institute of Biochemistry, Chemin des Boveresses, CH-1066 Epalinges, Switzerland.

0022-2631/79/0048-0141/$05.20

defining the metabolic cost of Na^+ transport and its thermodynamic implications [3]. Finally, physical factors such as stretch [29] or osmolarity [17], which can modify the basal rate of Na^+ transport, have been investigated in the toad bladder model.

The epithelium of the bladder is heterogeneous, and four cell types have been described [4, 22]: granular, mitochondria-rich, mucous (goblet), and basal cells. The first three cell types form a continuous sheet with their apical membrane facing the urinary lumen. The underlying basal cells are also heterogenous as shown in the accompanying paper [14]. They have no contact with the lumen and rest on the basement membrane. The physiological role of each cell type in the hormone-dependent transport of electrolytes has not been determined with certainty. Isolation and separation of these cells facilitate the characterization of functional differences and interrelationships, provided that dispersed cells retain the properties they have in intact tissue. Attempts to dissociate the toad bladder epithelium into single cells and to enrich for each cell type have been made by Scott *et al.* [25]. The separation was based on isopycnic centrifugation of isolated cells on discontinuous Ficoll gradients, and it was reported that almost pure granular and mitochondria-rich cell fractions were obtained. Degree of enrichment was not related to recoveries. Using the same procedure, Handler and Preston [10] were unable to discriminate vasopressin-induced adenylate cyclase in the MR and Gr fractions. Moreover, it was concluded in the latter study that cell viability was low and response to vasopressin variable as a consequence of cell damage generated by prolonged incubation in calcium-free medium (EDTA, 1 mM). To improve cell viability, we have developed a dissociation procedure based on collagenase digestion, calcium chelation with low EGTA concentration and mild shearing treatment. Separation was achieved by isopycnic centrifugation on continuous serum albumin gradients. Viability of cells was assessed by Trypan blue exclusion, reaggregation ability including formation of junctional complexes, and oxygen consumption in presence or absence of inhibitors such as ouabain and acetazolamide.

Materials and Methods

Reagents

Reagents were obtained from the following sources: chromatographically purified collagenase (*Clostridium histolyticum*), ~450 U/mg, from Worthington Biochemical Corp., Free-

hold[1], N.J.; bovine serum albumin (BSA) powder fraction *V* from Armour Pharmaceutical, Chicago, Ill.; gentamycin from Schering Corp., Fenilworth, N.J.; Instagel from Packard Instrument Company International, Zurich, Switzerland. Ouabain was from Sigma Chemical Co, St. Louis, and Merck, Darmstadt, Germany, and acetazolamide (free acid) was a gift from Lederle Co. All other chemicals were reagent grade.

Incubation Media

Perfusion medium (*A*) contained 90 mM NaCl, 3.5 mM KCl, 25 mM $NaHCO_3$, 0.5 mM $MgSO_4$, 0.5 mM KH_2PO_4, 1 mM $CaCl_2$, 6 mM glucose, 5 mg/liter gentamycin (220 mosmol, pH 7.4–7.5 under 95% O_2 – 5% CO_2 at 25 °C). Dissociation medium (*B*) contained 90 mM NaCl, 3.75 mM KCl, 25 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 14 mM glucose, 10 mM HEPES, 10 mg/liter gentamycin and complete aminoacid supplement [1] (236 mosmol, pH 7.4–7.5 under 95% O_2–5% CO_2 at 25 °C). Incubation medium (*C*) was similar to medium *B* but supplemented with 1 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 2% BSA (wt/vol). Culture medium (*D*) was amphibian medium purchased from Grand Island Biological Co., Grand Island, N.Y. All media were filtered through 0.22 μm Millipore filters (Millipore Corp., Bedford, Mass.), and all glassware was siliconized and sterilized before use.

Animals

Adult male and female *Bufo marinus* toads of Columbian origin were obtained from Charles P. Chase Co., Miami, Florida. Animals were kept at 25 °C in a terrarium with access to water maintained at 25 ± 0.5 °C and fed once a week with newborn mice. For each experiment 3 to 12 toads were killed by pithing and perfused through a heart puncture with oxygenated (95% O_2 – 5% CO_2) medium *A*. The hemibladders were then removed and rinsed in medium *A*. The epithelial cells were either scraped from the bladder with a microscope slide (scraped cell preparation) or dispersed according to the dissociation protocol described below.

Dissociation Protocol

A sequence of collagenase digestion, divalent cation chelation, and mechanical disruption as modified from Kraehenbuhl [13] and from Amsterdam and Jamieson [1] was used in the dissociation procedure of toad bladder epithelial cells. The detailed protocol is shown in Table 1. The dissociation was carried out at 25 °C under 95% O_2 and 5% CO_2 atmosphere. The different steps have been monitored under low magnification phase contrast or interference contrast optics and by recovery measurements based on DNA content as compared to that in scraped epithelial tissue. After perfusion (step 1), bladders were collected rinsed in medium *A* (step 2), and incubated for 45 min in medium *B* containing collagenase, 0.1 mM Ca^{++} and 1.2 mM Mg^{++} (step 3). The bladders were rinsed then with medium *B* containing 0.5 mM EGTA, no Ca^{++} and no Mg^{++}, and incubated for 15 min in this solution (step 4). At the end of incubation, the bladders were immersed in a Petri dish containing the same solution (medium *B* + EGTA), rubbed slowly between two fingers, and the epithelium was removed by passing a blunt microscope slide one or two times

[1] Some commercial batches are heavily contaminated with clostripain, trypsin-like enzymes and phospholipase. Collagenase was further purified according to the procedure of Gunther *et al.* [8].

Table 1. Sequence of dissociation and separation of toad bladder epithelial cells

Steps	Treatment	Medium composition	Time (min)	DNA recovery
1	Perfusion	Medium *A* (1 mM Ca^{++}, 0.5 mM Mg^{++})	20	
2	Collection of bladders	As in step 1	5	100[a]
3	Enzyme digestion	Medium *B*+0.1 mM Ca^{++}, 1.2 mM Mg^{++} Collagenase (200 U/ml)	45	
4	Calcium chelation	Medium *B*+0.5 mM EGTA	15	
5	Epithelium scraping (2 strokes with blunt microscope slide)	As in step 4	0.5	98
6	Pipetting	As in step 4	5	
7	Filtering (nylon gauze 20 μm mesh)	As in step 4	2	67
8	Washes	Medium *B*+0.5 mM EGTA +4% BSA	10	
9	Freshly dissociated cell suspension ($3–5 \times 10^7$ cells/ml)	Medium *B*+1 mM EGTA	2	62
10	Isopycnic centrifugation on a 0 to 35% BSA gradient $10{,}000 \times g_{avg}$, 20 °C	Medium *B*+0.5 mM EGTA	30	
11	Washes	As in step 4	10	34
12	Final suspension (2.0×10^6 cells/ml)	Medium *B*+1 mM Ca^{++}		

[a] 100% was estimated from measurements of wet wt of intact bladder and from the DNA-wet wt ratio (Table 2).

with slight pressure on the serosal side of the immersed bladder (step 5). A fine suspension of epithelial cells was obtained, which was pipetted up and down a siliconized Pasteur pipette (step 6)[2]. Diluted cell suspensions (5×10^5 cells/ml) were filtered under slight vacuum through a 20 μm mesh nylon gauze, mounted on a Sterifil 47-mm septic filtering system (Millipore Corp., Bedford, Mass.) (step 7). The cell suspension was centrifuged at 1,000 rpm (Centrifuge Sorvall GLC-2, rotor type HL-4), resuspended in 10 ml of medium *B*+EGTA, and layered over two 8-ml cushions of medium *B* EGTA 0.5 mM+4% BSA (step 11). After centrifugation at 1,000 rpm for 5 min, the intact cells formed a packed pellet whereas cell debris were found in the supernatant which was discarded.

Cell Separation by Isopycnic Centrifugation

Separation of cell populations was carried out on three types of gradients (step 10): discontinuous Ficoll or BSA gradients (8 ml) (densities: 1.088, 1.067, 1.035 and 1.017)

[2] This step was required to disrupt tight and gap junctions [1].

and continuous BSA gradients (10 ml, 6.7 cm) ranging from 35% to 0% BSA (density: 1.100 to 1.010). The gradients were generated in cellulose nitrate tubes (SW40 Ti Beckman rotor). Ficoll and BSA were dissolved in medium *B* containing 0.5 mM EGTA and the densities adjusted using a standard curve relating the concentration to the weighed density. 35% BSA solutions were dialized against medium *B*+EGTA until the sodium concentration and the pH were the same as medium *B*. Discontinuous gradients were generated by layering successively 2 ml of the four previously mentioned BSA or Ficoll solutions. The freshly dissociated cells were suspended in 2 to 4 ml of medium *B*+EGTA (final concentration: $3\text{–}5 \times 10^7$ cells/ml) (step 9, Table 1) and layered on top of continuous or discontinuous gradients. The tubes were spun for 30 min $10{,}000 \times g_{avg}$ at 25 °C. Some discontinuous gradients were spun at $20{,}000 \times g_{avg}$ for 45 min. Isopycnic centrifugation on discontinuous Ficoll or BSA density gradients (step 10–12, Table 1) yielded four main fractions. The continuous BSA gradients were harvested in 4 fractions after removing the sample volume: a very light fraction (VL) (2.9 ml) with a mean density $\bar{\rho}$ of 1.025, the light fraction (*L*) (2 ml) $\bar{\rho} \simeq 1.045$, a heavy fraction (*H*) (3 ml) $\bar{\rho} \simeq 1.065$, and a pellet (*P*) (2 ml) $\bar{\rho} \simeq 1.085$. The cell fractions and the pellets were then harvested and washed in medium *B*+EGTA (step 11) before DNA measurements and counting.

Recovery Measurements

Recoveries at each step of the dissociation procedure were determined by measuring the DNA content according to the fluorometric method of Kissane and Robbins [12]. To relate the final DNA recovery to the initial epithelial DNA content of intact tissue and to compare some of our values with those of the literature, the mean DNA and protein contents were determined in intact hemibladders of six animals and/or in epithelial cells scraped from the paired hemibladders of the same animals (Table 2). Protein was determined by the method of Lowry *et al.* [19].

Table 2. Reference values for wet weight, dry weight, DNA and protein content of whole tissue and scraped epithelium

	Whole tissue (1 hemibladder)		Scraped epithelial cells (1 hemibladder)	
Wet wt (ww) mg ww/hemibladder	278 ±31	(12)	75 ±8	(12)
Dry wt/ww	0.14 ± 0.01	(6)	0.11 ±0.01	(6)
DNA content				
mg DNA/mg ww	0.019± 0.004	(6)	0.022 ±0.003	(6)
mg DNA/hemibladder	0.516± 0.085	(6)	0.166 ±0.026	(6)
Protein content				
mg protein/mg ww			0.0219±0.0015	(16)
mg protein/hemibladder			1.63 ±0.08	(16)
Protein/DNA ratio mg protein/mg DNA			9.90 ±0.51	(16)

Data are expressed as mean value ± SE. In parantheses, number of experiments.

Morphological Methods

Pieces of intact toad bladder were fixed in 2% formaldehyde-2% glutaraldehyde in 0.1 M Na cacodylate, pH 7.4, for 2 hr at room temperature. Dissociated cells were fixed by mixing 1 vol of cell suspension with 2 vol of the above fixative. Tissue blocks and dispersed cells were postfixed for 1 hr at 4 °C, in 1% OsO_4 buffered with 0.1 M Na cacodylate to pH 7.4, washed once with Veronal-acetate buffer, pH 7.4, and stained in blocks for 1 hr with 0.5% uranyl acetate in Veronal-acetate buffer, pH 5. The tissue or cells were dehydrated in ethanol and propylene oxide and embedded in Epon. For light microscopy, 0.5 μm Epon sections were stained with 1% methylene blue in 1% Na borate, examined, and photographed with a Zeiss Photomicroscope II. Phase contrast and differential interference microscopy were performed on the same microscope to monitor the different steps in the dissociation and separation procedure.

Oxygen Consumption

QO_2 was measured at 25 °C with a Clark electrode in a Gilson-Oxygraph K-IC. QO_2 was measured in aliquots of cells ($5–25 \times 10^6$ cells in medium *B* or *C)* for at least 10 min for each experimental period and was linear with time up to 30 min. Cell losses (magnetic stirring) were estimated by measuring DNA content in the supernatant and did not exceed 2%. QO_2 measurements were related to DNA content and expressed in nmol O_2/hr·μg DNA. Ouabain (10^{-2} M) and DNP (5×10^{-3} M) were dissolved in medium *B*, whereas acetazolamide (2.5×10^{-2} M) was dissolved in 0.025 N NaOH.

Results

Reference Values

In order to monitor recoveries and yields in our dissociation procedure, it was necessary to determine the epithelial cell content in intact bladders. Hemibladders were weighed and the epithelium was scraped. The efficiency of scraping was analyzed morphologically, and no epithelial cells remained associated with the submucosa. Dry weight, DNA, and protein content were measured, and values are summarized in Table 2.

Light and Electron Microscopy

Intact tissue. The epithelium which lines the urinary lumen of the toad bladder is composed of an heterogeneous cell population including ~55% granular cells, ~7% mitochondria-rich cells, ~8% goblet (mucous) cells, and ~30% basal cells (Table 3, upper part). These values were obtained from light and electron micrographs of 10 different toads.

Table 3. Distribution of epithelial cell types in intact bladder and in cell fractions

	Gr cells		MR cells		G cells		MFR cells		Un cells		% cells from starting material		% DNA
Intact bladder[a]	55±5		7±1		8±2		20±3		10±3		100		100(86–114)[b]
Freshly dispersed cells[a]	56±5		5±3		5±3		21±3		11±4		62		64(57–71)
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	
Very light fraction[c]	19	0.3	27	0.4	0	0	50	0.75	4	0.05	1.5	0.5	0.5(0.5–1.5)
Light fraction[c]	65	1.6	12	0.3	0	0	19	0.5	4	0.10	2.5	1.0	1.0(1.0–7)
Heavy fraction[c]	82	61	3	2.0	2	1.5	3	2.5	9	7	74	25	25.7(24–33)
Pellet fraction[c]	48	10	3.5	0.7	18	4	4.5	1.0	26	6	22	7.5	7.5(7–12)
Total recovery of cells after centrifugation[c]		73		3.5		5.5		4.75		13		34	35 (30–39)

[a] Mean values expressed as percent of total epithelial cells ± SEM from seven toad bladders.

[b] Total DNA for a pool of 10 bladders was 2,560 µg ($n=3$). The ranges are indicated in parentheses.

[c] Values of a typical experiment. Columns A: percent of cells in the fractions. Columns *B*: percent of recovery from the gradient.

Only cells with visible nuclei were counted. Granular, mitochondria-rich, and goblet cells form a continuous monolayer with their apical membrane facing the urinary lumen; they are sealed at their apices by tight junctions which separate the urinary cavity from the intercellular compartments (Fig. 1). The fine structure of these cells has been well documented [4, 22]. The basal cells are heterogeneous and constitute two cell types: MFR cells and undifferentiated cells, as described in the accompanying paper [14]. The proportion of each cell type was quite constant when different fields were counted from the same bladder. Variations in the distribution of cell types occured from one animal to the next; for instance, the distribution of goblet cells varied from 1 to 15%.

Freshly dispersed cells. After dissociation and filtration on 20 µm mesh nylon gauze, a fine suspension of dispersed cells was obtained (Fig. 2) provided Ca^{++} was absent from the medium. Cell number was determined using a hemocytometer; in parallel samples the average DNA

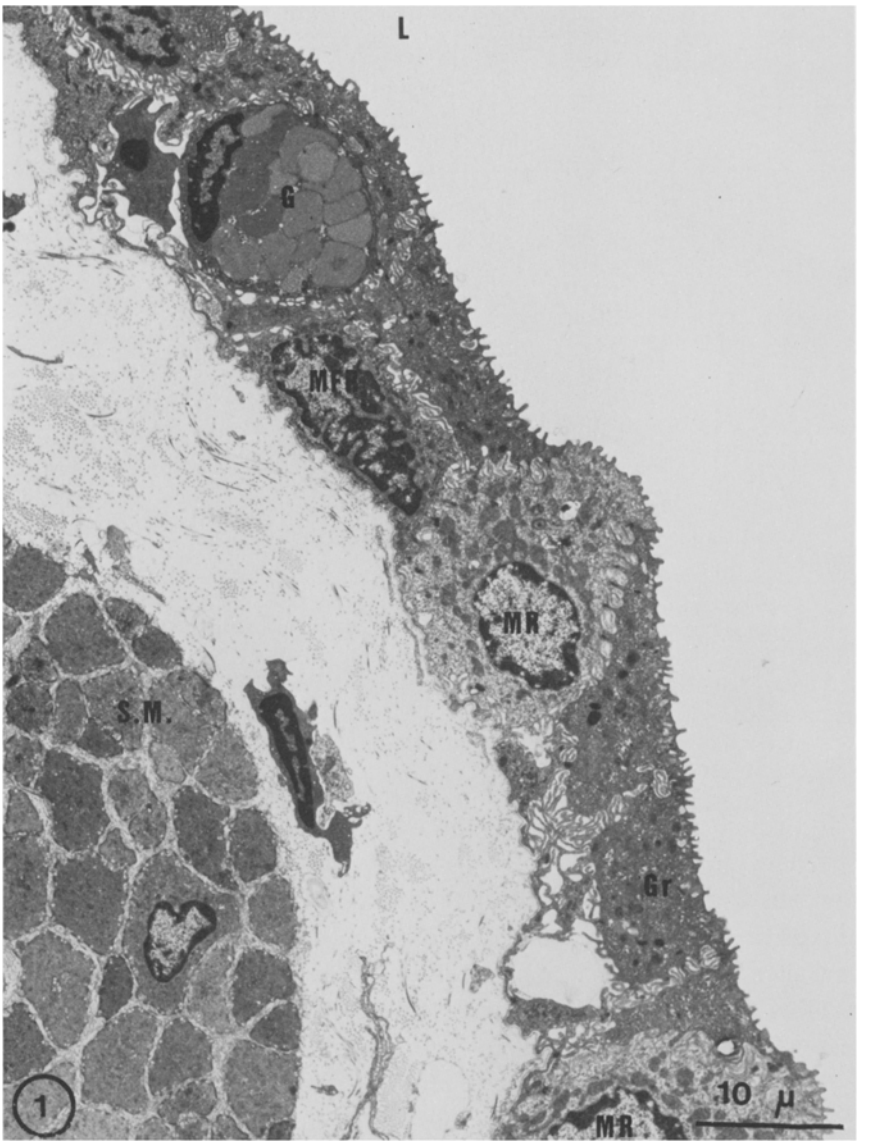
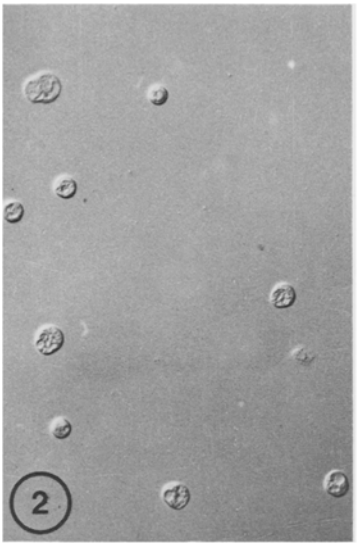
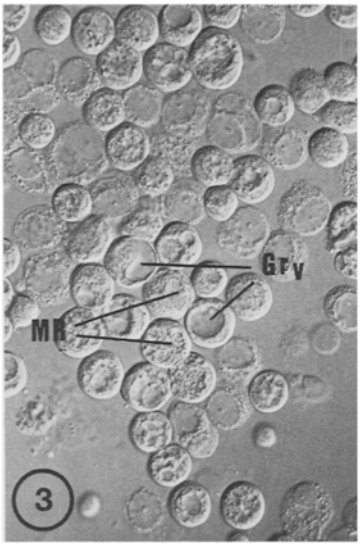


Fig. 1. Electron micrograph of a section cut through the bladder of *Bufo marinus*. The epithelium is composed of granular (*Gr*), mitochondria-rich (*MR*), goblet (*G*) cells which form a monolayer facing the urinary lumen (*L*). Microfilament-rich (*MFR*) cells are located basally. Smooth muscle cells (*SM*) are also present in this low-power micrograph. Stained with uranyl acetate and lead citrate. 2,000×

content was measured and a value of 13.2 ± 0.9 pg DNA per cell was found ($n = 39$), which is comparable to 11.3 in toad erythrocytes [2] and 9.7 in liver cells [5].

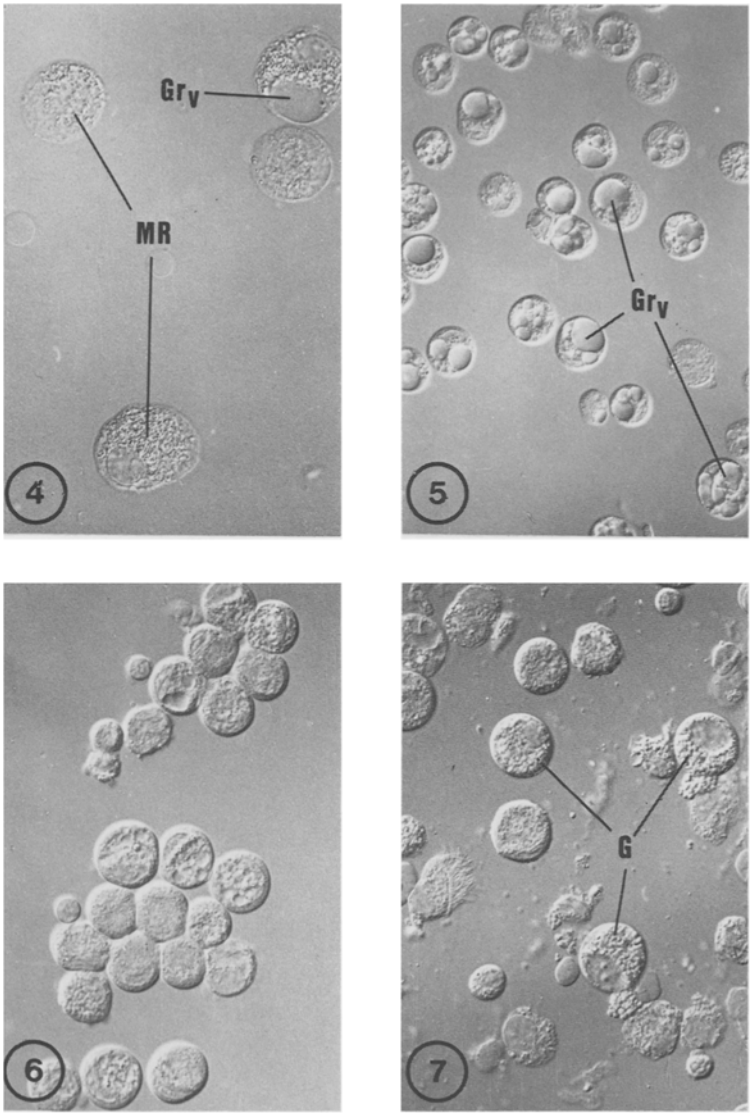




Figs. 2 and 3. Differential interference microscopy of living dispersed unfractionated cells. *Fig. 2:* Diluted cell suspension (10^5 cells/ml) indicating dispersion of epithelial cells. 100×. *Fig. 3:* Cells at higher density. Mitochondria-rich (*MR*) and vacuolated granular (*Grv*) cells can be identified. 300×

Aggregation was rapidly induced either by increasing the cell concentration (5×10^6 cells/ml) and/or by reintroducing Ca^{++} in the medium (Figs. 3, 6 and 10). Under interference contrast microscopy, MR-cells and G-cells were easily identified. The distribution of the different cell types in freshly dispersed cell preparations reflected that of intact tissue (Table 3). The cell counts were obtained from EM micrographs and a total of 2.485 cells were examined. About 70% of the cells excluded Trypan Blue.

Cell fractions: Dissociated cell suspensions were enriched in each cell type following an equilibrium density gradient separation adapted from Steimann and Cohn [26] (Table 1, steps 9–11). The cells, after centrifugation to equilibrium on a continuous BSA gradient, were separated into 4 main fractions: a VL fraction ($\rho \simeq 1.025$) enriched in MR-cells as visualized by interference contrast microscopy (Fig. 4), an *L* fraction ($\rho \simeq 1.045$) containing cells with large vacuoles (Fig. 5), an *H* fraction ($\rho \simeq 1.065$) with aggregated cells (Fig. 6), and a *P* fraction ($\rho \simeq 1.085$) enriched in goblet cells (Fig. 7). For each of the 4 fractions, the total number of cells, the cell DNA recovery and the cell types present as



Figs. 4–7. Differential interference microscopy of living dispersed cells fractionated on BSA gradients. *Fig. 4:* Very light fraction composed of cells containing numerous small dense bodies (*MR*-cells). 400×. *Fig. 5:* Light fraction consisting of cells containing large vacuoles (*Grv*) 300×. *Fig. 6:* Heavy fraction containing aggregated cells with no recognizable features. 300×. *Fig. 7:* Pellet was composed of cells with large granules (goblet cells) (*G*) and some cell debris 300×

observed on 5 μm Epon-embedded sections (light microscopy) and thin sections (electron microscopy) were determined and the results are summarized in Table 3. The 4 cell fractions, examined by electron microscopy

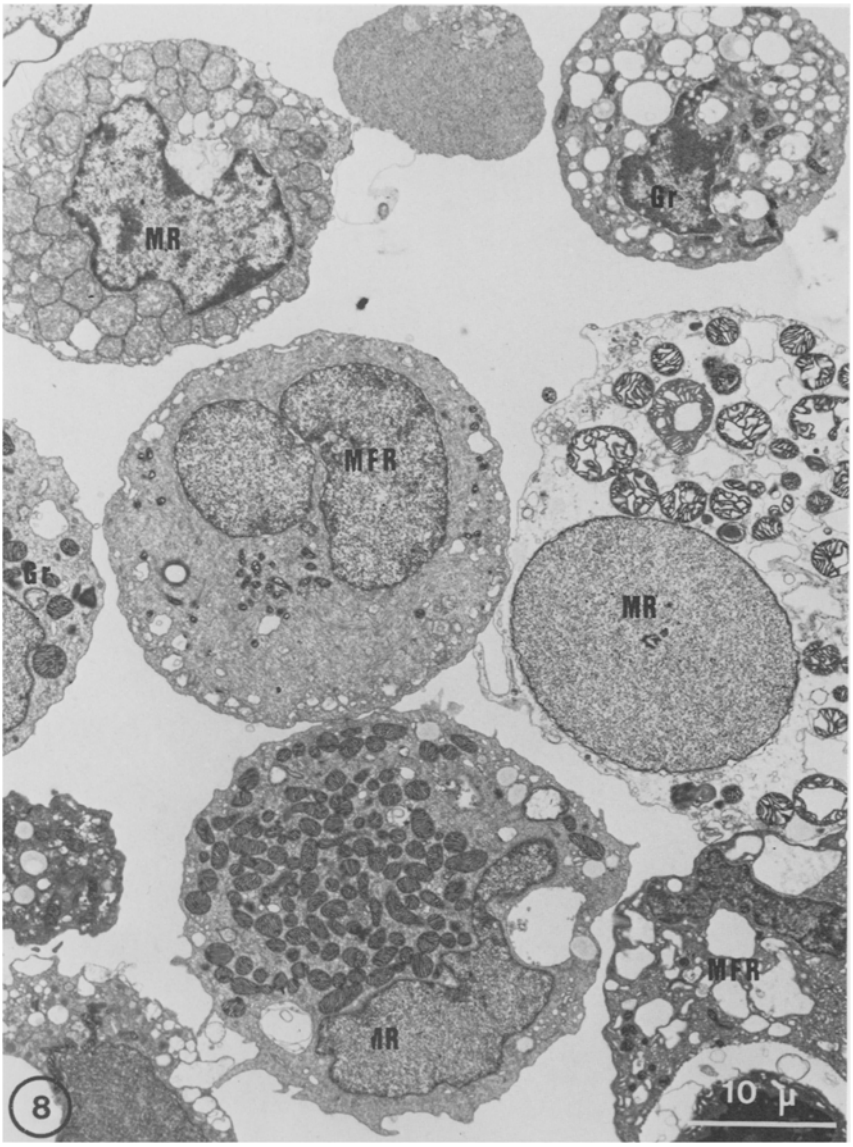


Fig. 8. Electron micrograph of a section through cells from the very light fraction. Several MR cells contain numerous mitochondria, whose appearance is heterogeneous from one cell to the other. MFR-cells are also present in this fraction. Stained with uranyl acetate and lead citrate. 2,500×.

had the following distribution of cell types: the *very light fraction* consisted of ~27% MR-cells, 50% MFR-cells and 19% vacuolated GR-cells (Figs. 4 and 8, Table 3). Some MR-cells were poorly preserved with vacuo-

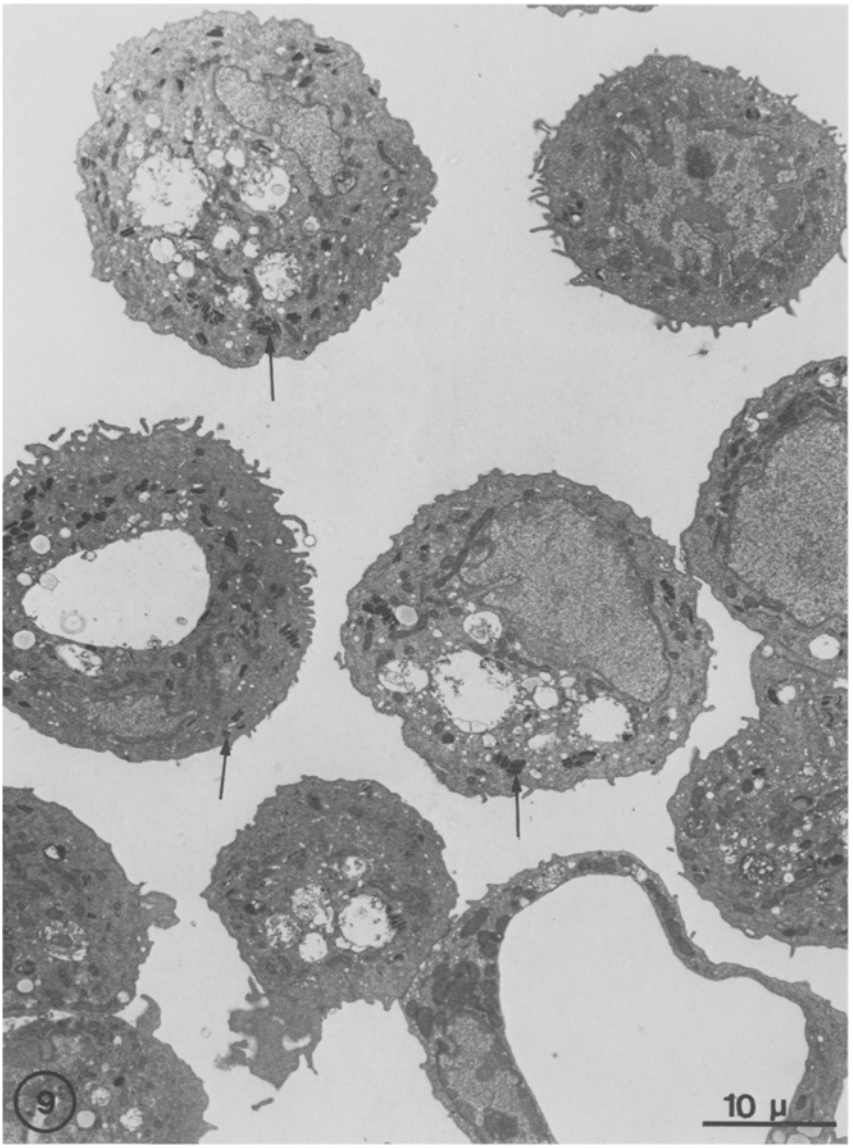


Fig. 9. Section through cells from the light fraction. The cells contain typical dense granules (arrows) and maintain polarity with microvilli restricted to one pole of the cell. Large vacuoles are present in the cytoplasm. Stained with uranyl acetate and lead citrate. 1,800 ×

lated mitochondria, as shown in Fig. 3, and could account for the Trypan Blue positive cells (< 10%). MFR-cells were identified by the presence of intermediate filaments which were usually running from the nucleus to one pole of the cells. 0.5–1.5% of the total cells applied on a continuous

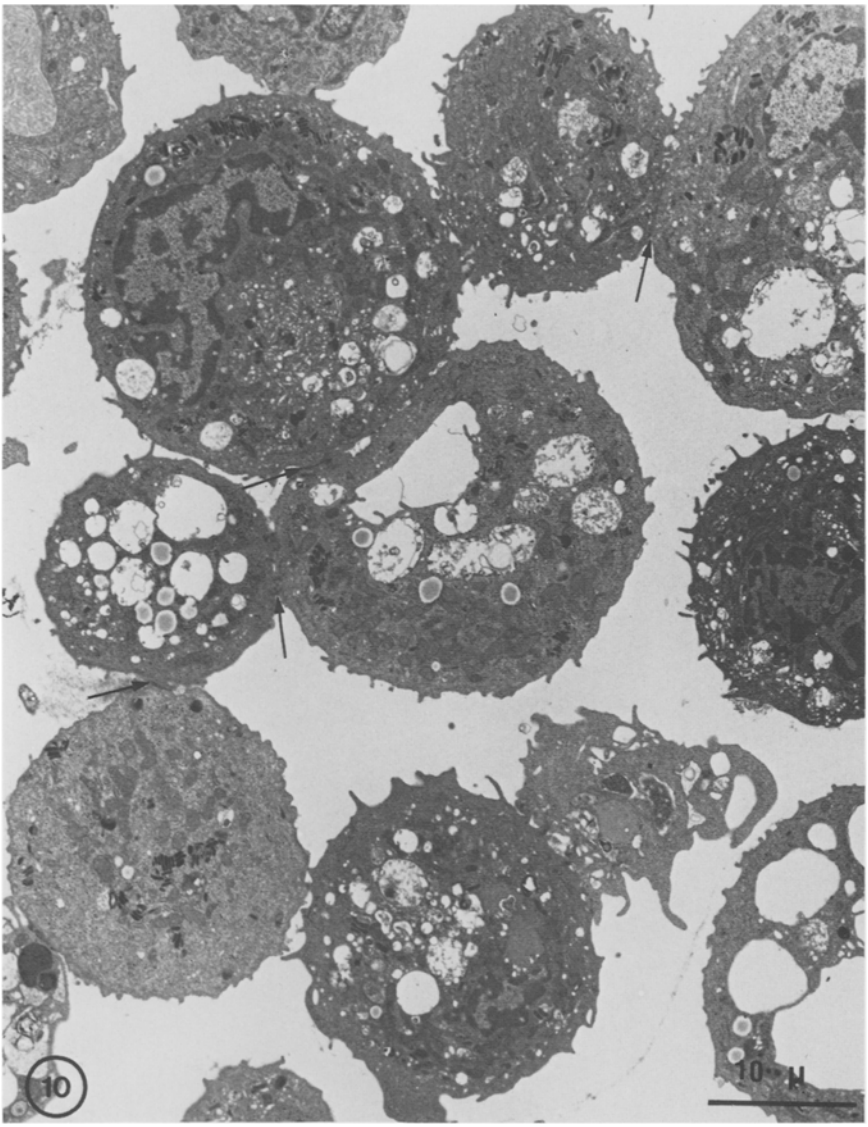


Fig. 10. Section through cells from the heavy fraction. Cells are aggregated, and junctional complexes (arrows) are seen between cells. Dense granules are found at the periphery of the cells. Stained with uranyl acetate and lead citrate. 2,000 ×.

BSA gradient was recovered in the very light fraction. The *light fraction* was composed of 65% vacuolated Gr-cells, 12% of MR-cells and 19% of MFR-cells representing 2.5% of the total cells recovered from the gradient (Fig. 9, Table 3). Less than 5% of the cells were Trypan Blue

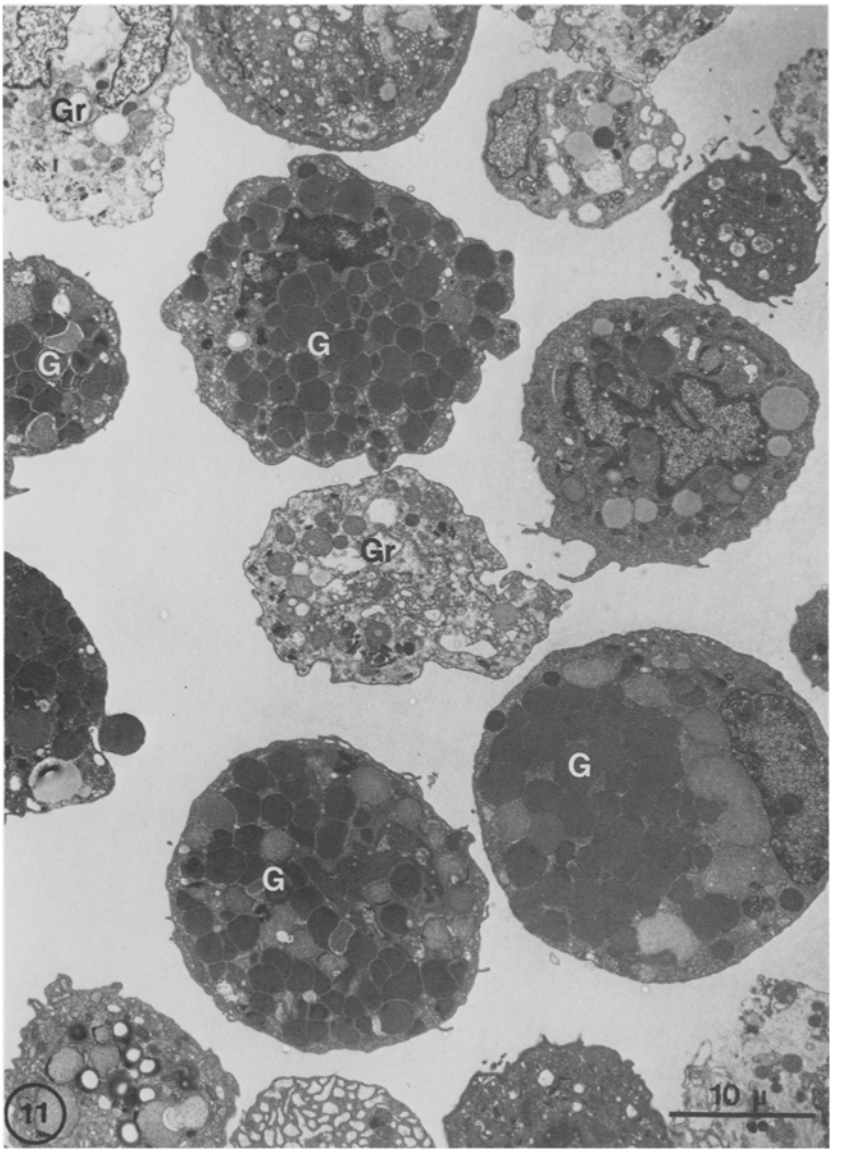


Fig. 11. Section through cells from the pellet. Typical goblet cells (*G*) are present together with granular cells. Stained with uranyl acetate and lead citrate. 2,000×

positive. Hemidesmosomes were frequently associated with the membrane surrounding the vacuoles. The *heavy fraction* contained 75% of the total cells recovered from the gradient, with less than 10% Trypan blue positive cells. It contained ~80% aggregated Gr cells with tight junctions and desmosomes joining two of more cells (Fig. 10). Smaller vacuoles were

present in their cytoplasm (Fig. 10). MR-cells, MFR-cells, G- and Un-cells represented less than 8% of the heavy fraction cell population (Table 3). *The pellet* represented ~20% of the total cells recovered from the gradient and consisted of 48% Gr cells, 18% of G-cells (Fig. 11) and 26% of Un-cells, which could not be identified by specific intracellular organelles (*see* Fig. 7 in accompanying paper [14]). Nucleated erythrocytes recovered in the pellet were not included in cell counts.

Reaggregation

At 25 °C unfractionated cells as well as fractionated cells reaggregated rapidly (~1.5 min) upon addition of Ca^{++} to the medium, whereas at 4 °C the cells remained dispersed, provided cell concentration remained lower than 5×10^5 cells/ml. Cells from heavy fractions were incubated 24 hr at a concentration of 10^6 cells/ml in medium *D*, fixed and processed for electron microscopy. Junctional complexes including tight junctions and desmosomes were readily observed between cells (Fig. 12). In addition, cell polarity was reestablished with numerous microvilli facing the incubation medium. Finally the large vacuoles present in dispersed granular cells were no longer detectable when cells were allowed to reaggregate and establish junctions. Cells from unfractionated preparations behaved similarly with formation of junctions between different cell types.

Recovery and Enrichment

The yield of washed freshly dissociated cells was 20 to 50% based on DNA content as compared to that of intact epithelium. From 1 g of fresh tissue $39 \pm 5 \times 10^6$ ($n = 10$) single cells were recovered. The major loss, as shown in Table 1, occurred during pipetting, filtering, and isopycnic centrifugation. As shown in Table 3, no selective loss of one given cell type was detected in freshly dispersed cell preparation when compared to intact bladder. After isopycnic centrifugation ~55% of the cells applied on a continuous gradient were recovered, with selective loss of MR- and MFR-cells. The distribution of cells in the heavy and light fractions varied from one experiment to another (Table 3, column 7). It may reflect the variable degree of cell aggregation which is function of cell concentration applied on the gradient. To compare the overall yield of our procedure with that of techniques described by others [25], freshly dispersed

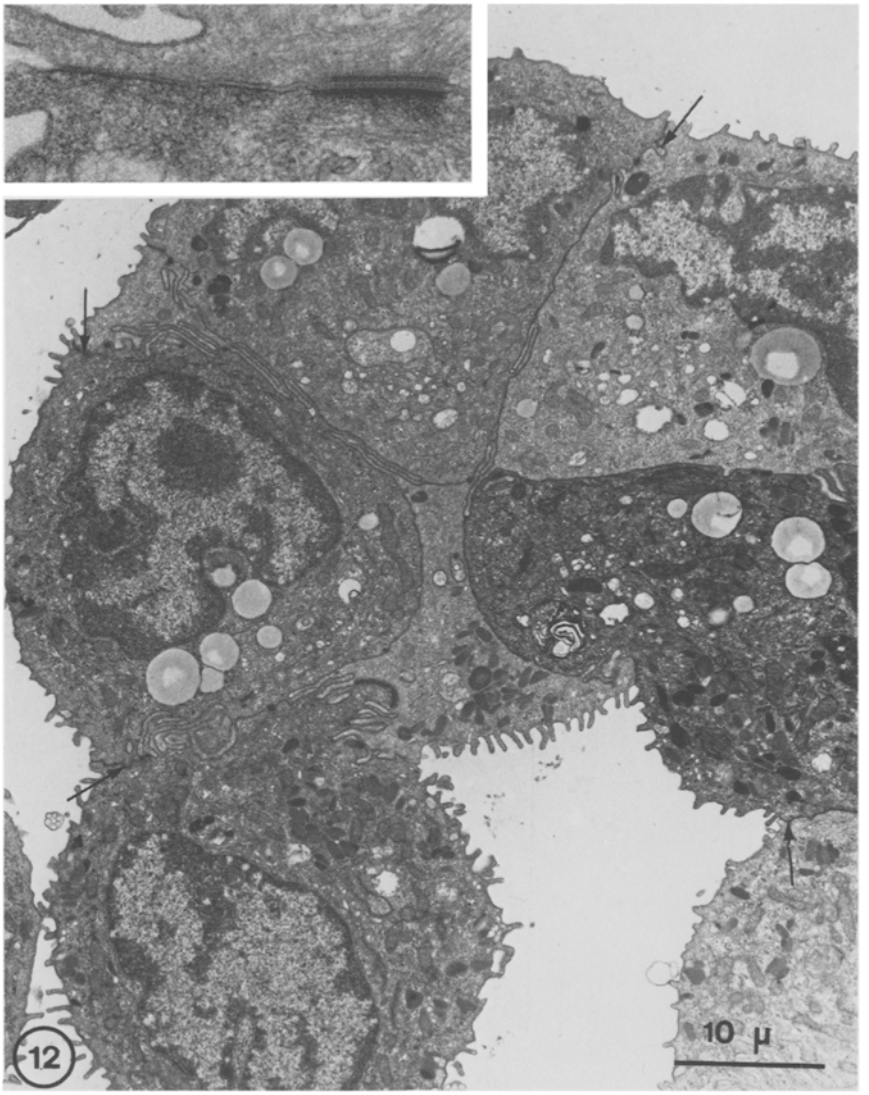


Fig. 12. Section through cells from the heavy fraction cultured 24 hr in medium *D* (*see Methods*). Aggregates of granular cells are present with junctional complexes including tight junctions (arrows). The cell exhibit polarity with microvilli facing the incubation medium. Stained with uranyl acetate and lead citrate. 2,000×. *Inset:* High magnification micrograph of aggregated cells, illustrating the presence of a tight junction and a desmosome. 10,000×

cells were submitted to centrifugation on discontinuous gradient in Ficoll. When we followed Scott's procedure, most cells were recovered in the pellet (74%). In addition, 50% of the cells contained in the very light fraction banding at the 1.017–1.035 interface were Trypan blue positive.

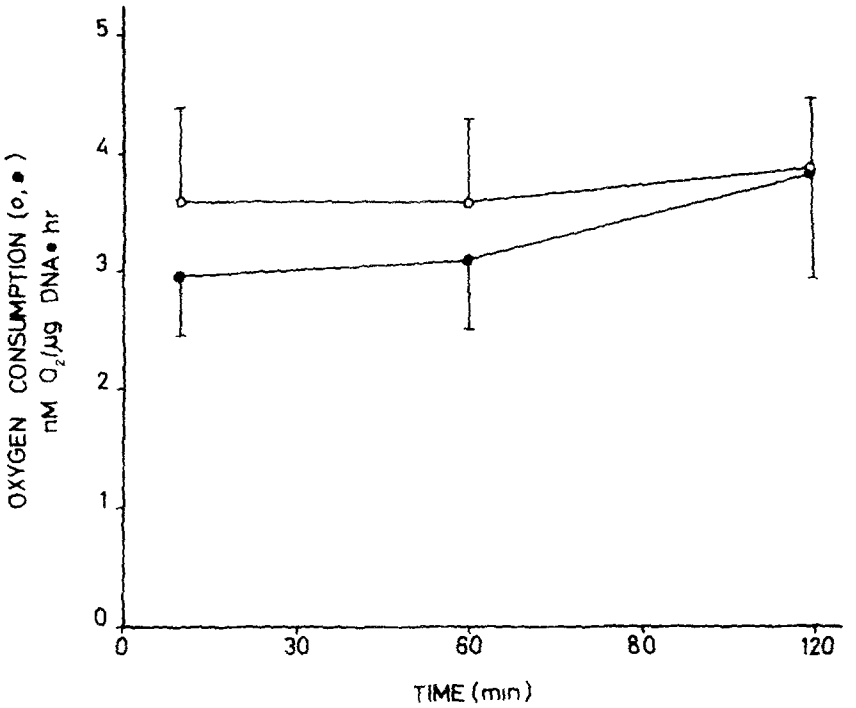


Fig. 13. Comparison of the oxygen consumption (QO_2) of scraped cells (–o–) and homologous pools of cells dispersed following the procedure described in methods (–•–). $n=5$ (±1 SE)

Oxygen Consumption

a) In scraped epithelium vs. dispersed or reaggregated cells. To assess the proportion of oxygen consumption contributed by the epithelium, we measured O_2 consumption in epithelial sheets of scraped bladders. The consumption in freshly dispersed cells was compared to that of scraped epithelium to determine the effects of collagenase, EGTA, and mechanical disruption.

Four pools of 8 toads were used: one third of each whole bladder was scraped and incubated further without any treatment; the remaining $^2/_3$ were dissociated. The scraped or dissociation cells were incubated at 25 °C for up to 120 min. Figure 13 shows that QO_2 of freshly dissociated cells at any time did not significantly differ from that of scraped cells. In addition, QO_2 remained constant as long as 120 min.

It was observed that aggregation of cells was dependent on Ca^{++} concentrations. QO_2 was determined for cells incubated in presence or

Table 4. Effects of removal or addition of calcium on oxygen consumption of dispersed cells

	QO_2 (I)	QO_2 (II)	Fractional change II/I
	0.5 mM EGTA or EDTA	1.5 mM $CaCl_2$	
Mean ± SEM ($n=6$)	2.08 ± 0.06	2.66 ± 0.13	1.760 ± 0.045[a]
	1 mM Ca^{++}	1.5 mM EGTA	
Mean ± SEM ($n=3$)	3.31 ± 0.6	2.70 ± 0.36	0.833 ± 0.056[b]

Freshly dispersed cells were incubated for 120 min in Medium *B* supplemented with 2% BSA in presence of 0.5 mM EGTA (or EDTA) or 1.5 mM $CaCl_2$.

QO_2 was measured or described for 15 min (*I*) and then $CaCl_2$ or EGTA was added directly to the 2.0 ml chamber for another 15 min (*II*).

[a] $p<0.005$.

[b] $p<0.1$.

absence of Ca^{++} and results are summarized in Table 4. When cells were preincubated in 0.5 mM EGTA or EDTA, there was a 28% increase in QO_2 upon addition of Ca^{++} ($p<0.005$, $n=6$). In the reverse situation a 17% decrease in QO_2 was observed which, however, did not reach the level of significancy ($p<0.1$, $n=3$).

b) In enriched cell fractions. Total oxygen consumption was measured in each fraction following isopycnic centrifugation and compared to that of unfractionated cells. In addition, the metabolic cost of Na^+ and H^+ transport was estimated by measuring the ouabain and acetazolamide sensitive QO_2. In a few experiments, QO_2 was also measured in presence of dinitrophenol (50 μM) to determine the maximum oxygen consumption (uncoupled state). In all cases DNP increased the basal rate of oxygen consumption by 20 to 50%. Results are summarized in Fig. 14. The QO_2 in the very light, light, and pellet fractions was ~twofold higher than in unfractionated cells. In contrast, total QO_2 in the heavy fractions was only $^2/_3$ of that of unfractionated cells and one third of that of the light and very light fractions. All these differences were significant ($p<0.01$).

In preliminary experiments, it was shown that 1 mM ouabain was sufficient to inhibit more than 95% of the Na^+ transport estimated by short-circuit current in intact tissue. At this concentration, the effect of oubain was slowly but totally reversible (data not shown). Ouabain-

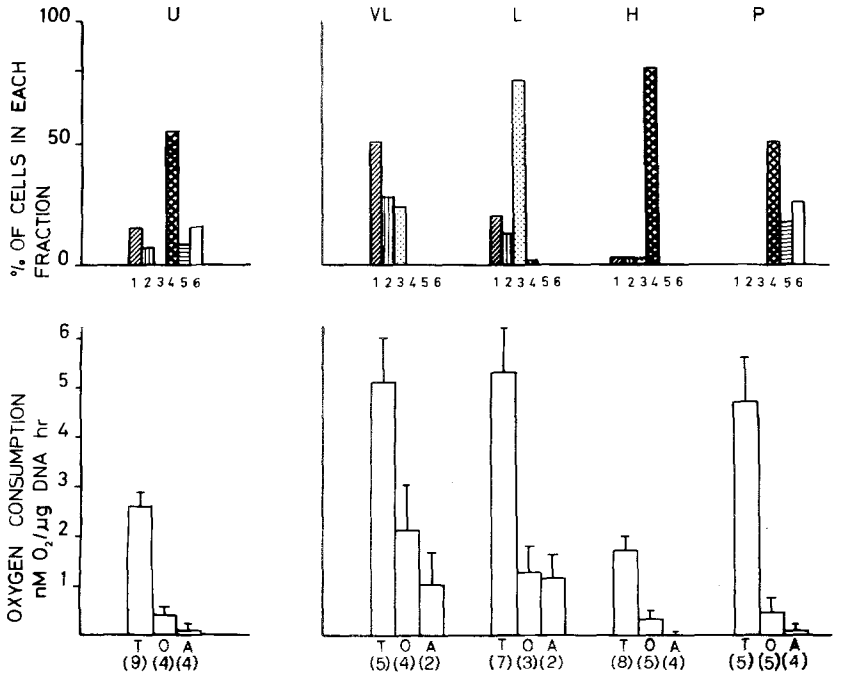


Fig. 14. *Lower panels:* Total QO_2 (*T*), ouabain-sensitive QO_2 (*O*) and acetazolamide-sensitive QO_2 (*A*) in unfractionated cells (*U*) before density centrifugation (left panels) and in various fractions [very light (*VL*), light (*L*), heavy (*H*) and pellet (*P*)] collected after density centrifugation on a continuous 0–35% albumin gradient. Mean $\pm$ 1 SE and number of experiments are indicated. *Upper panels:* Distribution of various cell types in various fractions are represented following the results shown in Table 3. Form left to right the bars indicate (1) MFR-cells; (2) MR-cells; (3) vacuolated granular cells; (4) granular cells; (5) goblet cells; (6) undifferentiated cells

sensitive QO_2 accounted for 18% of total QO_2 in unfractionated cells but varied from 34% in the very light fraction to $\sim$18% in the light and heavy fraction and $\sim$12% in the pellet. Due to rather large experimental variations, the difference was significant only between the VL and *P* fractions ($p < 0.05$).

Acetazolamide-sensitive QO_2 was determined in each fraction. A concentration of 1 mM was shown to abolish H^+ transport measured by reversed short-circuit current as proposed by Ludens and Fanestil [20]. Acetazolamide-sensitive QO_2 accounted only for 3.5% of the total QO_2 in unfractionated cells (different from zero, $p < 0.01$), -0.1% in the pellet (not different from zero, $p < 0.8$) and -0.8% (not different from zero, $p > 0.5$) in the heavy fraction. As shown in Fig. 15, it represented $\sim$20% in the lighter fractions (VL+*L*), which represented a 20-fold increase over the heavy fractions (*H*+*P*) ($p < 0.05$).

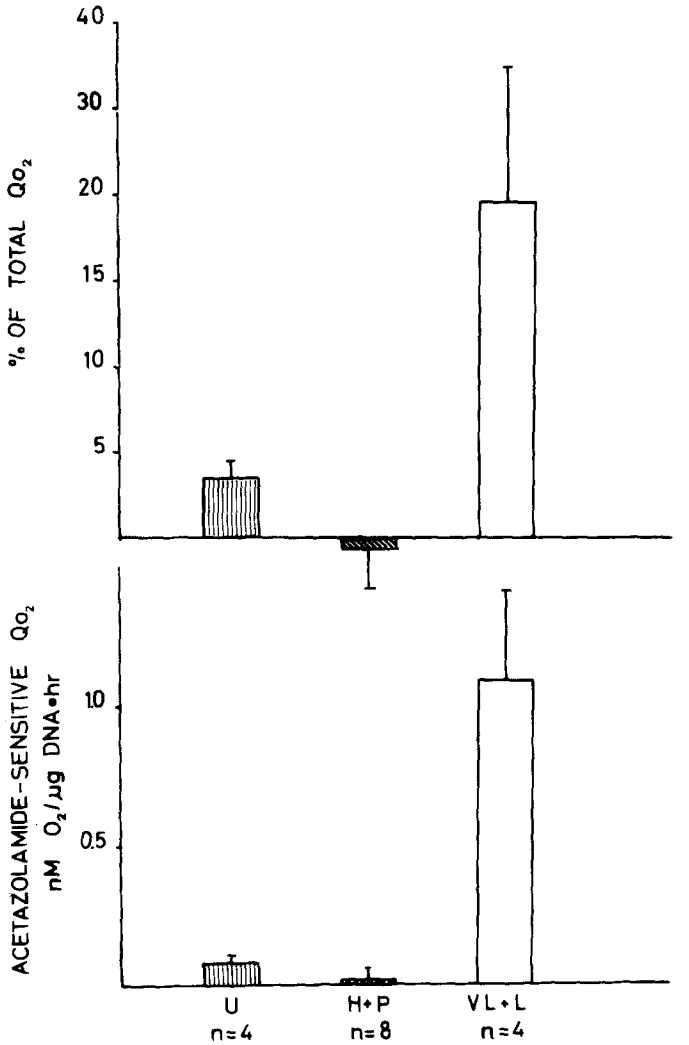


Fig. 15. Acetazolamide-sensitive QO_2 in unfractionated cell (*U*), very light and light fractions (*VL+L*), heavy and pellet fractions (*H+P*). *Upper panel:* acetazolamide-sensitive QO_2 as percent of total QO_2. *Lower panel:* acetazolamide-sensitive QO_2 in absolute value. Mean ± 1 SE are indicated

c) Time course of total QO_2, ouabain, and acetazolamide-senstive QO_2 in heavy fraction. To evaluate the effect of prolonged incubation on the level of oxygen consumption, QO_2 was measured in the heavy fraction 1 and 16 hr after dissociation. As shown in Fig. 16, total QO_2 and acetazolamide-sensitive QO_2 was steady over a 16 hr period, whereas ouabain-sensitive QO_2 decreased from 18 to 11% (not significant, $p > 0.2$). The

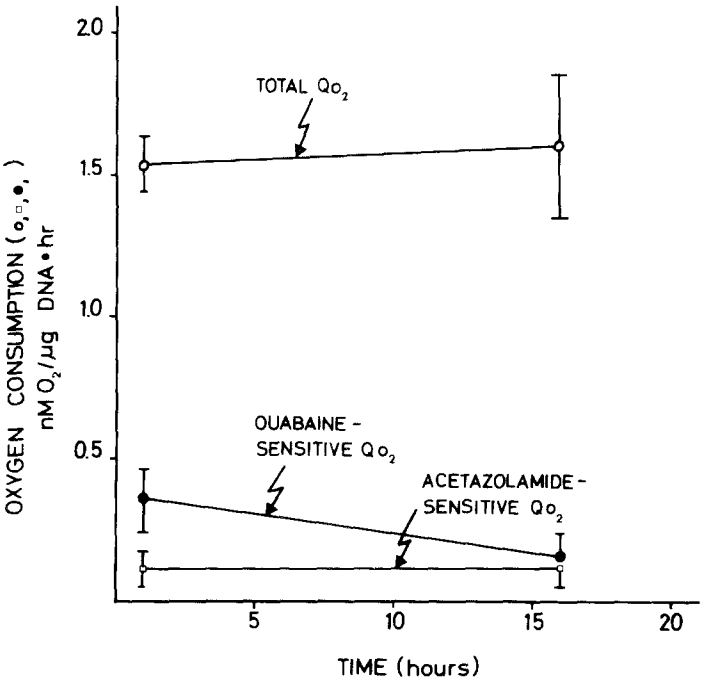


Fig. 16. Time course of total QO_2-, ouabain-, and acetazolamide-sensitive QO_2 in cells collected in the heavy fraction after density centrifugation. Mean ± 1 SE are indicated. $n=4$

total cell number and Trypan positive cells (< 10%) did not change during the assay. Due to insufficient cell number these determinations could not be performed in the light and very light fractions.

Discussion

This paper describes a dissociation and separation procedure which generates viable dispersed epithelial cells from the bladder of *Bufo marinus*. A dissociation procedure was reported by Scott *et al.* [25] which relied upon divalent cation chelation (EDTA) and mechanical disruption followed by separation of cell types using isopycnic centrifugation on discontinuous Ficoll gradients. In our hands, Scott's procedure yielded 4 fractions, most cells (74%) being recovered in the pellet. Each fraction, although enriched in a given cell type, was contaminated by other cell types. In addition, over 50% of the cells were Trypan blue positive and cell integrity was lost as visualized by electron microscopy. In order to increase total

recovery of dispersed cells and cell viability, a procedure modified from Jamieson and Amsterdam [1] and Kraehenbuhl [13] was used, including collagenase treatment, calcium chelation (EGTA) and gentle shearing. Separation of cell types was achieved by equilibrium density centrifugation on continuous BSA gradient. Recovery was monitored at each step of the dissociation protocol. Major cell loss occurred during filtering ($\sim 40\%$) and isopycnic centrifugation ($\sim 30\%$). Analysis of cell composition indicated a similar distribution of cell types in unfractionated dispersed cell preparations as compared to intact tissue. After centrifugation, there was selective loss of MR-cells ($\sim -40\%$) and MFR-cells ($\sim -75\%$) accompanied by a concomitant enrichment of granular cells ($\sim +30\%$) (Table 3). The cell composition of the 4 fractions recovered from the BSA gradients was analyzed morphologically. The very light fraction was enriched 4.5-fold in MR-cells over the unfractionated cell fraction, confirming results reported by Scott [25], but also 2.4-fold in MFR-cells. In addition, the fraction was contaminated by granular cells. The light fraction was also enriched in MR-cell to a lesser degree (twofold) and in granular cells which were characteristically vacuolated (Fig. 9). The heavy fraction was enriched in granular cells (1.5-fold). Contamination by other cell types was low, since the maximum theoretical enrichment is 1.8-fold. More than $^3/_4$ of the cells were recovered in this fraction, whereas light and very light fractions contributed less than 5% of total recovery. The remainder was recovered in the pellet which was enriched in goblet cells (3.6-fold) and undifferentiated cells (2.2-fold).

The overall recovery obtained with our dissociation and separation procedure varied between 30 and 40% and thus compares favorably with yields reported in mammalian system [1, 13, 24]. Cell viability in unfractionated and fractionated preparations was assessed by exclusion of vital dyes, oxygen consumption, and ability to reestablish cell junctions upon addition of calcium.

Up to 30% of freshly dispersed cells did not exclude Trypan blue. This high percentage of damaged cells remained constant after dissociation was completed, indicating that cell damage or death occurred during the dissociation steps (mechanical disruption). Damaged cells could be removed by density centrifugation. In all fractions recovered after isopycnic centrifugation including the pellet, less than 10% of the cells were Trypan blue positive. This suggests that lysis of damaged cells occurred during centrifugation.

Unfractionated and fractionated cells reaggregated upon addition of calcium in a time-, temperature-, and energy-dependent manner. As

shown in Fig. 12, junctional complexes, including desmosomes and tight junctions, reappeared. The formation of junctions was parallelled by the disappearance of vacuoles in granular and MR-cells (Fig. 12). Reaggregation was rapid (1–5 min) at 25 °C and did not occur at 4 °C. The QO_2 consistently and rapidly increased upon addition of calcium which induced reaggregation, and this process was reversible (Table 4). At steady state, however, the QO_2 of scraped cells (not exposed to enzymes or EGTA) which have intact junctional complexes, was similar to that of a paired pool of dispersed cells (Fig. 13). This suggests that increased oxygen consumption is a consequence of membrane reorganization including loss of vacuoles and formation of junctions and not an inherent property of dispersed cells.

Oxygen consumption is expressed on a cellular basis, i.e., cellular DNA instead of the classic dry wt basis [7, 18, 21][4]. When converting our data in μl O_2 per dry wt × hr, 1.5 μl O_2 mg dry wt · hr was found which is in the lower range of published values [11]. One should point out that an absolute estimate of QO_2 *per se* is not very meaningful, since several factors affect oxygen consumption, including subspecies differences [18], temperatures at which toads are kept before experiments, and incubation medium (composition, pH, osmolarity).

In cell preparations recovered from the BSA gradients, oxygen consumption remained steady for at least 16 hr (Fig. 13). The value reported in the heavy fraction, enriched in Gr cells (1.5-fold), is probably an overestimate of the true QO_2 of granular cells, since this fraction is still contaminated by MR-, MFR- and vacuolated Gr-cells, which all together have a threefold higher metabolic acitivity than cells in the heavy fraction. It is tempting to attribute such high metabolic activity to MR-cells, since the increase in oxygen consumption parallels enrichment of MR-cells in the light fractions. However, we have no way at the present time to rule out the potential role of MFR- and vacuolated Gr-cells in such high O_2 consumption rates. High rates of oxygen consumption in the two light fractions could also be a consequence of

[4] The dry wt includes both the cellular and the extracellular space dry wt. The contribution of dry wt of extracellular space, including variable amounts of salts and mucous (secreted by the goblet cells) may introduce significant errors when oxygen consumption is measured in preparation containing few cells ($< 2 \times 10^6$) and compared to that of scraped cells. However, in order to compare our values with those reported in the literature [7, 21] we have converted μl O_2/hr · μg DNA hr into μl O_2/dry wt/hr using the dry wt/DNA ratio as calculated from Table 1. Dry wt was not corrected for the contribution of the extracellular space, thus the ratio of 20 is probably an underestimate of the "true" dry wt/DNA ratio.

uncoupling of oxydative phosphorylation. Two lines of evidence speak against a high degree of uncoupling. First, ouabain and acetazolamide inhibited QO_2 to a greater extent than in any other fractions. Second, QO_2 was further stimulated by addition of dinitrophenol. Finally, by density centrifugation we have separated a subpopulation of granular cells containing large vacuoles. This could be interpreted as cell damage. However, these cells exclude Trypan blue. By selectively labeling the luminal membrane (data not shown) of the epithelium, we have observed that luminal plasma membranes are internalized in vacuolated granular cells following dissociation. As already mentioned, this process is reversible. The role and the reasons of appearance of these vacuoles remain unknown. In intact epithelia (frog skin), scalloped vacuoles have been observed and it has been suggested that they play a role in the transepithelial transport of Na^+ [27].

The metabolic cost of two well-characterized transport processes has been estimated by using specific inhibitors, ouabain for Na^+ transport and acetazolamide for H^+ transport. This approach is indirect; no other direct methods to measure the cost of specific cellular functions are available at the present time. Lipton and Edelman [18] have shown in the toad bladder that approximately 25% of the total QO_2 was inhibited by ouabain. Specificity was demonstrated by the absence of inhibition in Na^+ free medium. In the present study, ouabain-sensitive QO_2 represented 18% of total QO_2 in unfractionated cell preparations and is the integral of the values estimated in the various cell fractions. Thus, ouabain-sensitive QO_2 accounted for 34% of total QO_2 in the VL fraction, but only 11% in the pellet. Since our separation protocol does not yield homogeneous cell population, it is not possible to correlate closely ouabain-sensitive QO_2 with a given cell type. There were also striking differences in acetazolamide-sensitive QO_2 with a 20-fold increase in the lighter fractions compared to the heavier fractions. These results suggest that H^+ transport takes place in MR-cells and/or MFR-cells. MR-cells are good candidates since carbonic anhydrase has been histochemically located in MR-cells of frog skin [28].

In conclusion, the present study provides evidence that the toad bladder epithelium can be dissociated into dispersed cells and that isopycnic centrifugation produces fractions enriched in various cell types. The cells are viable and display heterogeneity in metabolic activities such as Na^+ and H^+ transport. Further purification, however, will require additional separation procedures. Preliminary results indicate differential lectin binding to various cell types and techniques using lectins to separate cell

types are available [23]. Finally, in the course of this study it was found that the population of basal cells is heterogeneous. Morphological and some functional properties of basal cells are described in the following paper [14].

We wish to thank Dr. E.L. Cooper for revising the manuscript. This study was supported by grants from the Swiss National Science Foundation #3-514.075, #3-122.077, and 3-731.076 and from the Fritz Hoffmann La Roche Foundation #156.

References

1. Amsterdam, A., Jamieson, J.D. 1974. Studies on dispersed pancreatic exocrine cells. I. Dissociation technique and morphologic characteristic of separated cells. J. Cell Biol. **63**:1037
2. Bachmann, K. 1970. Specific nuclear DNA amounts in toads of the genus *Bufo*. *Chromosoma* **29**:365
3. Caplan, S.R., Essig, A. 1977. A thermodynamic treatment of active sodium transport. *In:* Current Topics in Membranes and Transport. F. Bronner and A. Kleinzeller, editors. p. 145. Academic Press, New York
4. Choi, J.K. 1963. The fine structure of the urinary bladder of the toad, *Bufo marinus*. *J. Cell Biol.* **16**:53
5. Conger, A.D., Clinton, J.H. 1973. Nuclear volumes, DNA contents and radiosensitivity in whole body irradiated amphibians. *Radiat. Res.* **54**:69
6. Edelman, I.S. 1975. Mechanism of action of steroid hormones. *J. Steroid Biochem.* **6**:147
7. Gatzy, J.T., Berndt, O. 1968. Isolated epithelial cells of the toad bladder. Their preparation, oxygen consumption, and electrolyte content. *J. Gen. Physiol.* **51**:770
8. Gunther, G.R., Schultz, G.S., Hull, B.E., Alicea, H.A., Jamieson, J.D. 1977. Functional pancreatic acini prepared using collagenase with low clostripain activity. *J. Cell Biol.* **75**:368*a*
9. Handler, J.S., Orloff. 1973. The mechanism of action of antidiuretic hormone. *In:* Handbook of Physiology. Sect. 8, Chap. 24, pp. 791–814. Am. Physiol. Soc. Washington, D.C.
10. Handler, J.S., Preston, A.S. 1976. Study of enzymes regulating vasopressin-stimulated cyclic AMP metabolism in separated mitochondria-rich and granular epithelial cells of toad urinary bladder. *J. Membrane Biol.* **26**:43
11. Hays, R.M., Singer, B., Malamed, S. 1965. The effect of calcium withdrawal on the structure and function of the toad bladder. *J. Cell Biol.* **25**:195
12. Kissane, J.M., Robins, E. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem* **233**:184
13. Kraehenbuhl, J.P. 1977. Dispersed mammary gland epithelial cells. I. Isolation and separation procedures. *J. Cell Biol.* **72**:406
14. Kraehenbuhl, J.P., Pfeiffer, J., Rossier, M., Rossier, B.C. 1979. Microfilament-rich cells in the toad bladder epithelium. *J. Membrane Biol.* **48**:167
15. Leaf, A., Anderson, J., Page, L.B. 1958. Active sodium transport by isolated toad bladder. *J. Gen. Physiol.* **41**:657
16. Leaf, A., Hays, R.M. 1962. Permeability of the isolated toad bladder to solutes and its modification by vasopressin. *J. Gen. Physiol.* **45**:921

17. Lipton, P. 1972. Effect of changes in osmolarity on sodium transport across isolated toad bladder. *Am. J. Physiol.* **222**:821
18. Lipton, P., Edelman, I.S. 1971. Effects of aldosterone and vasopressin on electrolytes of toad bladder epithelial cells. *Am. J. Physiol.* **221**:733
19. Lowry, O.H., Rosebrough, N.J., Fahr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
20. Ludens, J.H., Fanestil, D.D. 1972. Acidification of urine by the isolated urinary bladder of the Columbian toad. *Am. J. Physiol.* **226**:1321
21. McKnight, A.D.C., DiBona, D.R., Leaf, A., Civan, M.M. 1971. Measurement of the composition of epithelial cells from the toad urinary bladder. *J. Membrane Biol.* **6**:108
22. Peachey, L.D., Rasmussen, H. 1961. Structure of the toad's urinary bladder correlated to its physiology, *J. Biophys. Biochem. Cytol.* **10**:529
23. Reisner, Y., Ravid, A., Sharon, N. 1976. Use of soybean agglutinin for the separation of mouse B and T lymphocytes. *Biochem. Biophys. Res. Commun.* **72**:1585
24. Romrell, L.J., Coppe, M.R., Munro, D.R., Ito, S. 1975. Isolation and separation of highly enriched fractions of viable mouse gastric parietal cells by velocity sedimentation. *J. Cell Biol.* **65**:428
25. Scott, W.N., Sapirstein, V.S. 1974. Partition of tissue functions in epithelia: Localization of enzymes in "mitochondria-rich" cells of toad urinary bladder. *Science* **184**:797
26. Steinman, R.M., Cohn, Z.A. 1974. Identification of a novel cell type in peripheral lymphoid organs of mice. *J. Exp. Med.* **139**:380
27. Voûte, C.L., Mollgard, K., Ussing, H.H. 1975. Quantitative relationship between active sodium transport, expansion of endoplasmic reticulum and specialized ("scalloped sacs") in the outermost living cell layer of the frog skin epithelium. *J. Membrane Biol.* **21**:273
28. Voûte, C.L., Thummel, J., Brenner, M. 1975. Aldosterone effect in the epithelium of the frog skin – a new story about an old enzyme. *J. Steroid Biochem.* **6**:1175
29. Walser, M., Butler, S.E., Hammond, V. 1969. Reversible stimulation of Na^+ transport in the toad bladder by stretch. *J. Clin. Invest.* **48**:1714